

Inactivation of Caspase-1 in Rodent Brain: A Novel Anticonvulsive Strategy

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Summary: *Purpose:* Cytokines and related inflammatory mediators are rapidly synthesized in the brain during seizures. We previously found that intracerebral administration of interleukin-1 (IL-1)- β has proconvulsant effects, whereas its endogenous receptor antagonist (IL-1Ra) mediates potent anticonvulsant actions in various models of limbic seizures. In this study, we investigated whether seizures can be effectively inhibited by blocking the brain production of IL-1 β , by using selective inhibitors of interleukin-converting enzyme (ICE/caspase-1) or through caspase-1 gene deletion.

Methods: Caspase-1 was selectively blocked by using pralnacasan or VX-765. IL-1 β release was induced in mouse organotypic hippocampal slice cultures by proinflammatory stimuli [lipopolysaccharide (LPS) + adenosine triphosphate (ATP)] and measured with enzyme-linked immunosorbent assay (ELISA). IL-1 β production during seizures was measured in the rat hip-

pocampus by Western blot. Seizures were induced in freely moving mice and rats by intrahippocampal injection of kainic acid and recorded by EEG analysis.

Results: Caspase-1 inhibition reduced the release of IL-1 β in organotypic slices exposed to LPS+ATP. Administration of pralnacasan (intracerebroventricular, 50 μ g) or VX-765 (intraperitoneal, 25–200 mg/kg) to rats blocked seizure-induced production of IL-1 β in the hippocampus, and resulted in a twofold delay in seizure onset and 50% reduction in seizure duration. Mice with caspase-1 gene deletion showed a 70% reduction in seizures and an approximate fourfold delay in their onset.

Conclusions: Inhibition of caspase-1 represents an effective and novel anticonvulsive strategy, which acts by selectively reducing the brain availability of IL-1 β . **Key Words:** Cytokines—Epilepsy—Inflammation—Interleukin-1 β —Glial—Hippocampus.

Inflammatory mediators are rapidly induced in rodent brain both in glia and in neurons during seizures (1–7). In particular, intracerebral application of interleukin-1 β (IL-1 β) prolongs the duration of electrographic (EEG) and behavioral seizures in rodents (3,6). Conversely, IL-1Ra, a naturally occurring antagonist of IL-1 β (8), mediates powerful anticonvulsant effects (4,6), and mice overexpressing IL-1Ra in astrocytes show a dramatically reduced susceptibility to seizures (6,9). Recently, the threshold to seizure induction in experimental models of febrile convulsions, was shown to be decreased by intracerebral IL-1 β injection and raised in IL-1 receptor-deficient mice or after delivery of IL-1Ra (10,11). These findings indicate that an increase in IL-1 β levels in the brain, as occurs during seizures, has proconvulsant effects.

This evidence prompted us to study whether preventing the brain production of IL-1 β during seizures results in significant anticonvulsive effects. This goal was achieved by using selective inhibitors or gene deletion of caspase-1 (12–14), the key enzyme specifically involved in the production of the releasable and biologically active form of IL-1 β .

MATERIALS AND METHODS

Experimental animals

Animals were housed at a constant temperature (23°C) and relative humidity (60%) with free access to food and water and a fixed 12-h light/dark cycle. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L.n.116, G.U., suppl.40, Feb. 18, 1992; UK legislation under the 1986 Animals Scientific Procedures Act) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Accepted February 17, 2006.

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doi: 10.1111/j.1528-1167.2006.00590.x

Rats

Male Sprague–Dawley rats (250–280 g) were purchased from Charles River (Calco, Italy).

Mice

Caspase-1 knockout (KO) mice and their wild-type strain (C57BL6/SV129, ~24 g) were kindly supplied by Dr. W. Wong (BASF Bioresearch Corporation, Worcester, MA, U.S.A.). Wild-type and KO animals were bred separately as homozygous groups from breeding couples of the same genetic background and at the time of the study were at generation 12 and 15, respectively.

Caspase-1 KO mice have been extensively characterized previously: they develop normally and are healthy and fertile; they are highly resistant to the lethal effects of lipopolysaccharide (LPS), and their macrophages exhibit a severe defect in the production of mature IL-1 β (15).

Placement of cannula and electrodes for EEG recordings

Rats

Rats were surgically implanted with cannulae and electrodes under stereotaxic guidance as previously described (3). In brief, rats were deeply anesthetized by using Equithesin (1% phenobarbital and 4% chloral hydrate; 3 ml/kg, intraperitoneally, i.p.). A ground lead was positioned over the nasal sinus, and two screw electrodes were placed bilaterally over the parietal cortex. Bipolar nichrome wire-insulated electrodes (60 μ m) were implanted bilaterally into the dorsal hippocampus (septal pole) at the following coordinates from bregma: mm, nose bar –2.5, AP –3.5, L \pm 2.4 and 3.0 below dura mater. A 22-gauge guide cannula was unilaterally positioned on top of the dura and glued to one of the depth electrodes for intrahippocampal injection of kainic acid. In some rats, an additional guide cannula was unilaterally positioned on top of the dura mater for intracerebroventricular (icv) injection of pralnacasan (in mm from bregma, nose bar –2.5; AP –1; L +1.5). The electrodes were connected to a multipin socket (March Electronics, Bohemia, NY, U.S.A.) and, together with the injection cannula, were secured to the skull by acrylic dental cement.

Mice

Mice were anesthetized by using 5% halothane (Concord Pharmaceuticals Ltd, Dunmow, Essex, U.K.) with oxygen as the carrier gas at a flow rate of 0.4 L/min. Anesthesia was maintained by inhalation of 1.5–2% halothane with oxygen. A ground lead was positioned over the nasal sinus. The stainless-steel recording electrode of an ETA-F20 small-animal telemetry device (Data Sciences International, St. Paul, MN, U.S.A.) was unilaterally implanted into the septal pole of the dorsal hippocampus (in mm from bregma, nose bar 0; AP –1.9; L –1.5 and 1.5 below dura mater), with the transmitter body being placed sub-

cutaneously on the animal's flank. A 22-gauge injection cannula was attached to the depth electrode and positioned on top of the dura mater for the intrahippocampal injection of kainic acid. The experiments were performed \geq 3 days after surgery to allow the animals to recover.

EEG recordings and injection of drugs

The procedures for recording the EEG and intracerebral injection of drugs in freely moving rats or mice have been previously described (3,6).

Kainic acid

Kainic acid (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in 0.5 μ l phosphate-buffered saline (PBS, pH 7.4) and injected in the mouse (14 ng) or rat (40 ng) dorsal hippocampus unilaterally (1 μ l/min), by using a needle protruding 1.5 or 3.0 mm, respectively, from the guide cannula.

This dose of kainic acid was chosen to induce recurrent EEG epileptic activity composed of ictal episodes (EEG seizures) and spiking activity in 100% of animals without mortality.

Pralnacasan and VX-765

Pralnacasan (Vertex Pharmaceuticals, Inc., Cambridge, MA, U.S.A.) was dissolved in 20% cremophor (25 μ g in 4 μ l, the maximal concentration soluble in the injected volume) and injected icv in rats or mice, 45 min and 10 min before intrahippocampal injection of kainic acid. In preliminary experiments, pralnacasan was also administered in a single dose (12.5 or 25 μ g in 4 μ l), 45 min before kainic acid. VX-765 (25, 50, 200 mg/kg; Vertex Pharmaceuticals) was dissolved in 20% cremophor and injected ip in rats once a day for 3 consecutive days. On the fourth day, rats received VX-765, 45 min and 10 min before intrahippocampal injection of kainic acid. Respective controls were similarly injected with vehicle before kainic acid.

The correct positioning of the injection needle and electrodes was verified by Nissl staining of 40- μ m cryostat coronal brain sections in all the animals, 24 h after kainic acid injection (not shown). In mice, no evidence of neuronal cell loss was observed in any experimental condition; in rats treated with kainic acid, we observed the typical pattern of pyramidal cell loss restricted to CA3 area, as previously reported in detail (3,16). The unspecific damage restricted to the insertion of injection needle and the electrode tracks was similar in control and experimental animals. All pharmacologic experiments were carried out between 9.00 a.m. and 1.00 p.m.

Seizure assessment and quantification

Rats

EEG seizures induced by intrahippocampal injection of kainic acid in rats have been extensively described

(3). In brief, a 30-min recording was done before kainic acid injection to assess the basal EEG pattern. Recording was stopped after 180 min from kainate injection, a time at which seizure activity was absent for ≥ 30 min in all the animals. Ictal episodes were characterized by high-frequency and/or multispikes complexes and/or high-voltage synchronized spikes simultaneously occurring in the injected hippocampus. Spiking activity was typically observed after seizures subsided (3). The EEG recording of each animal was analyzed visually by two independent investigators blinded to the treatments to detect any activity different from baseline. Seizure activity was quantified by counting the time to seizure onset (elapsed from kainic acid injection to the occurrence of the first EEG seizure) and the total number and duration of seizures (counted by summing the duration of every ictal episode during the EEG recording period) (3). Shortly after its administration, kainic acid induced stereotyped behaviors such as sniffing and gnawing. "Wet-dog shakes" were often observed shortly after kainate injection and during seizures.

Mice

EEG was acquired by remote telemetry. The signal from the recording depth electrode was transmitted by radiofrequency and captured by a receiver pad (Data Sciences) under the cage of each animal. This signal was relayed to a computer that sampled and recorded the analogue output by using Dataquest A.R.T. Analog (Data Sciences). The EEG patterns were recorded and visualized by using Data-pac 2K2 (RUN Technologies, Laguna Hills, CA, U.S.A.). A baseline recording of ≥ 30 minutes was acquired before drug treatments. Recording was stopped after 180 min from kainate injection, a time at which seizure activity was absent for ≥ 30 min in all the animals. Seizure activity was quantified as in the rat.

Western blot analysis

Different groups of rats ($n = 3$ in each treatment group) were treated icv with pralnacasan or its vehicle given alone, or before intrahippocampal application of kainic acid (see earlier). Ninety minutes after the onset of EEG seizures, when pralnacasan effect on seizures was evident (see Results), experimental rats and their controls were decapitated. The injected hippocampi were dissected out at 4°C and homogenized in 20 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA, 5 mM EGTA, 1 mM Na-vanadate, 2 $\mu\text{g}/\mu\text{l}$ aprotinin, 1 $\mu\text{g}/\mu\text{l}$ pepstatin, and 2 $\mu\text{g}/\mu\text{l}$ leupeptin (30 mg tissue/150 μl homogenization buffer). Total proteins (150 μg per lane; Bio-Rad Protein Assay, Bio-Rad Labs, Munich, Germany) were separated by using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10% acrylamide, and each sample was run in duplicate. Proteins were transferred to Hybond nitrocellulose membranes by electroblotting. For immunoblotting, we used anti-rat IL-1 β (0.2 $\mu\text{g}/\text{ml}$; Abcam Limited, Cambridgeshire, UK) or anti-

mouse IL-18 (1:250, kindly provided by Dr. Dinarello) rabbit polyclonal antibodies. Immunoreactivity was visualized with enhanced chemiluminescence (ECL; Amersham, U.K.) by using peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Sigma) as secondary antibodies. Densitometric analysis of immunoblots was done to quantify the changes in protein levels (AIS image analyzer; Imaging Research Inc., St. Catharines, Ontario, Canada) by using film exposures with maximal signals below the photographic saturation point. Optical density values in each sample were normalized by using the corresponding amount of α -actin.

Organotypic hippocampal slice cultures

Hippocampal slice cultures were prepared from 7-day-old C57BL/6 mice (Charles River, Calco, Italy), according to the interface culture method (17) with some modifications (18). In brief, mice were killed by decapitation, the brains removed under sterile conditions, and the two hippocampi isolated and cut in transverse sections at 350 μm , using a McIlwain tissue chopper. Individual slices were placed in ice-cold Gey's balanced salt solution (GBSS; Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 25 mM D-glucose (Merck, Germany); then they were placed on semiporous inserts membranes (6 slices on each membrane; Millipore Corp., Bedford, MA, U.S.A.) and transferred to 6-well culture trays (Corning Costar, Corning, NY, U.S.A.). Each membrane was immersed in a well containing 1 ml culture medium composed of 50% Opti-MEM, 25% heat-inactivated horse serum, and 25% Hank's balanced salt solution (HBSS) (Gibco BRL, Life Technologies Ltd., East Kilbride, Lanarkshire, Scotland, U.K.) and supplemented with 25 mM D-glucose. The cultures were incubated for 2 weeks with 5% CO_2 and 95% atmospheric air at 33°C , and medium was changed twice a week; no antibiotics or antimetabolites were used. On the day of the experiment, the culture medium was replaced with 1 ml of chemically defined, serum-free Neurobasal medium with 1 mM L-glutamine and B27 supplement (Gibco BRL, Life Technologies Ltd.).

Inflammatory stimuli were provided to organotypic slice cultures by preexposure to 10 ng/ml LPS (*Escherichia coli* serotype 055:B5; Sigma-Aldrich) for 3 h followed by 3 h incubation with 1 mM ATP (Sigma-Aldrich). Previous evidence in human monocytes has shown that this protocol is required for inducing both the production and the release of the mature and biologically active form of IL-1 β (19). To investigate the effect of caspase-1 inhibitors on IL-1 β production and subsequent release, slice cultures were preexposed to 0.1–10 μM pralnacasan or VX-765 for 2 h followed by coexposure with LPS for 3 h and LPS+ATP for a further 3 h.

Organotypic slices not exposed to drugs were used as controls in every experiment. At the end of the

experiments, tissue was pooled from six wells per experimental group, whereas the respective culture media were collected separately. All samples were stored at -70°C until assayed by ELISA.

ELISA

Hippocampal slices were homogenized in 0.5 ml ice-cold PBS (pH 7.4) containing a mixture of protease inhibitors (Roche Biochemical, Indianapolis, IL, U.S.A.), and 2% of NP-40 (Sigma-Aldrich). The homogenates were centrifuged, and the supernatants were collected to measure IL-1 β in the tissue.

IL-1 β was measured in tissue and medium by using an ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.) containing an antibody selective against murine IL-1 β that recognises both pro-IL-1 β and the mature form of IL-1 β . The cytokine was measured according to manufacturer's suggested protocol. Absorbance was read at 405 nm. The detection limit was $<3\text{ pg/ml}$.

Statistical analysis of data

Data are represented as the mean \pm SEM (n, number of individual samples). The effects of treatments were analyzed by two-way ANOVA followed by Fisher's test (for Western blot data), or by one-way ANOVA followed by Bonferroni's test (for organotypic cultures), or by one-way ANOVA followed by Tukey's test (for seizure analysis).

RESULTS

Effects of caspase-1 inhibition on hippocampal IL-1 β

In vitro evidence

It has been well established in cells of the immune system that LPS transcriptionally activates the IL-1 β gene, resulting in enhanced production of IL-1 β . However, in the presence of a second stimulus such as ATP, caspase-1 is activated enzymatically to process pro-IL-1 β to the mature and releasable IL-1 β form (17 kDa) (19). We applied this protocol to mouse organotypic slice cultures to study the effects of caspase-1 inhibitors on the levels and release of IL-1 β in hippocampal tissue.

Figure 1A and B shows that in organotypic hippocampal slice cultures, ATP is required for inducing a massive IL-1 β release in the presence of LPS, as previously shown in human monocytes (19). Thus LPS alone increased IL-1 β release into the culture medium by ~ 20 -fold ($p < 0.01$), whereas addition of ATP increased IL-1 β release by ~ 60 -fold compared with control untreated slices (panel A, $p < 0.01$ vs. LPS alone). Two hours of preincubation with pralnacasan or VX-765 reduced in a dose-dependent manner the LPS+ATP-mediated release of IL-1 β in the medium, reaching the levels observed when slices were incubated with LPS alone (panel A). Maximal 70% inhibition was reached with a concentration of $1\text{ }\mu\text{M}$ for both inhibitors. In the corresponding tissue extracts, LPS

or the coexposure to LPS+ATP increased IL-1 β levels to a similar extent ($p < 0.01$ vs. control slices; panel B) and pralnacasan or VX-765 did not affect LPS+ATP induced increase (panel B). The basal levels of IL-1 β in the medium and in the tissue of control slices (not exposed to any treatment) were barely or not detectable and did not differ from those measured in the presence of ATP or pralnacasan and VX-765 alone. Although the ELISA method used to measure IL-1 β in the tissue and in the medium does not permit distinguishing between pro-IL-1 β and the mature 17-kDa cytokine, it is well established that $>90\%$ of the releasable form of IL-1 β consists of the 17-kDa mature form, whereas the tissue content is mostly represented by pro-IL-1 β (20).

In vivo evidence

Figure 1C and D shows the effect of pralnacasan on IL-1 β levels induced in the rat hippocampus by seizures. Western blot analysis allowed distinguishing between the immature (pro-IL-1 β , 30 kDa) and the mature and releasable form of IL-1 β (17 kDa) (panel D). Seizures increased pro-IL-1 β by 84% and the mature and releasable form of IL-1 β by 20%. Anticonvulsant doses of pralnacasan (see later) fully reversed the effect of kainic acid-induced seizures on the mature, releasable form of IL-1 β ($p < 0.01$ vs. kainic acid), producing a total reduction of 50% below basal levels; consequently, pro-IL-1 β was increased by $\sim 50\%$ above the levels induced by seizures ($p < 0.01$ vs. kainic acid). In control hippocampi, pralnacasan alone did not significantly affect pro-IL-1 β while reducing mature IL-1 β levels by 30% below basal values (panel C).

IL-18 immunoreactivity was also assessed by using Western blot analysis because this cytokine is a substrate of caspase-1 (21). However, no signal was detected in hippocampal homogenates either in basal conditions or during seizure activity. The lack of signal was not due to failure of the Ab, because our Ab detected a specific band of 18 kDa when tested against $1\text{ }\mu\text{g}$ of human or rodent recombinant IL-18.

Effects of caspase-1 inhibition on kainic acid-induced seizures

Pralnacasan

Figure 2A shows the effect of two consecutive icv injections of pralnacasan on kainic acid-induced seizures in freely moving rats ($25\text{ }\mu\text{g}$ in $4\text{ }\mu\text{l}$ given 45 and 10 min before kainic acid injection). Pralnacasan significantly delayed the time to seizure onset by twofold ($p < 0.01$) and reduced the number of seizures by 20% ($p < 0.01$) and the total time spent in EEG seizures by 40% ($p < 0.01$). The average duration of seizures was also significantly reduced by 25% by pralnacasan (Vehicle, min, 1.6 ± 0.09 ; Pralnacasan, $1.2 \pm 0.07^{**}$, $n = 8$; $^{**}p < 0.01$ by Student's *t* test). Pralnacasan did not affect seizures when given in a

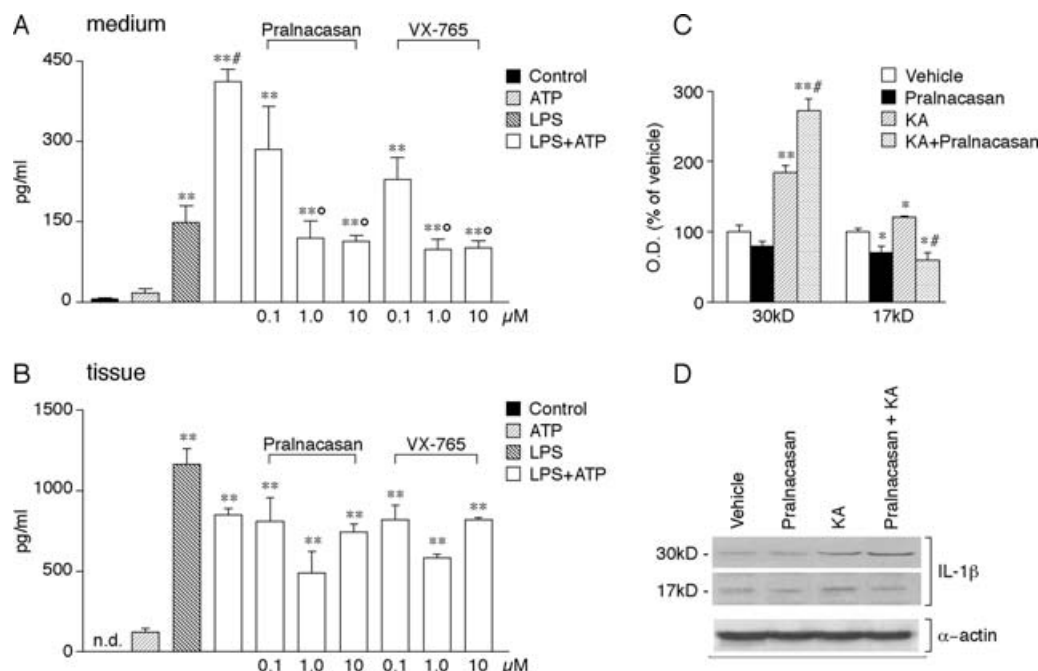


FIG. 1. Effects of pralnacasan and VX-765 on hippocampal IL-1 β levels **A, B:** Bargrams show IL-1 β levels (mean \pm SEM, $n = 6$) in mouse organotypic hippocampal slice culture media (**A**) and extracts (**B**), as assessed by ELISA. Slices were exposed for 3 h to 10 ng/ml lipopolysaccharide (LPS) followed by 3 h coincubation with 1 mM adenosine triphosphate (ATP), or they were incubated for 6 h with LPS alone. Slices were preincubated with pralnacasan or VX-765 (0.1, 1, and 10 μ M) for 2 h and for a subsequent 3 h with LPS, followed by 3 h exposure with LPS+ATP. Control cultures were not exposed to drugs. ** $p < 0.01$ versus control; # $p < 0.01$ versus LPS; ° $p < 0.01$ versus LPS+ATP by one-way ANOVA followed by Bonferroni's test ($F = 45.4$). **C, D:** Bargrams show the IL-1 β levels (mean \pm SEM, $n = 3$) measured in the rat hippocampus in the various experimental conditions, as assessed by Western blot. **C:** Quantification of the optical density (OD) of the IL-1 β protein bands of 30 kDa (pro-IL-1 β) and 17 kDa (mature IL-1 β) normalized to the corresponding α -actin levels (**D**). Data are expressed as percentage of normalized OD values measured in vehicle-treated rats. * $p < 0.05$; ** $p < 0.01$ vs. vehicle; # $p < 0.01$ vs. KA; IL-1 β (30 kDa): $F(3, 6) = 53.8$; IL-1 β (17 kDa): $F(3, 10) = 10.6$ by two-way ANOVA followed by Fisher test. **D:** Representative Western blots of pro-IL-1 β (30 kDa) and mature (17 kDa) IL-1 β in the rat hippocampus in the various experimental conditions. Vehicle: control rats injected with cremophor icv + PBS intrahippocampally (ih); pralnacasan: rats injected with pralnacasan (2 \times 25 μ g icv, 45 and 10 min before PBS ih); KA: rats injected with cremophor icv + 40 ng kainic acid ih; KA+pralnacasan: rats injected with pralnacasan 2 \times 25 μ g icv, 45 and 10 min before kainic acid ih. All rats were killed 90 min after the onset of kainic acid seizures or after the injection of PBS (vehicle and pralnacasan groups). Each sample was run in duplicate (150 μ g protein).

single administration (12.5 or 25 μ g in 4 μ l given 45 min before kainic acid) or at doses $< 25 \mu$ g (12.5 μ g in 4 μ l given 45 and 10 min before kainic acid; data not shown). Pralnacasan inhibited seizure duration by 52% and 38% of control values during the first and second hour of EEG recordings, respectively, while its effect faded away during the third hour of recording. Pralnacasan did not affect spiking activity (not shown).

VX-765

Figure 2B shows the effect of systemic administration of VX-765 on kainic acid-induced seizures. VX-765 at doses of 25, 50, and 200 mg/kg significantly delayed the time to seizure onset by 1.5- to twofold ($p < 0.01$), reduced the number of seizures by 40% ($p < 0.01$) and the total time spent in EEG seizure activity by 30 to 50% ($p < 0.01$). No differences in spiking activity were observed (not shown).

The extent of neuronal cell loss induced by kainic acid in the CA3 area of the injected hippocampus was similar in

pralnacasan- or VX-765-treated rats and their respective controls (not shown).

Seizure susceptibility in caspase-1 knockout (KO) mice

Figure 3B shows that the occurrence of seizures induced by intrahippocampal injection of 14 ng kainic acid was significantly delayed by approximately fourfold ($p < 0.01$) in caspase-1 KO mice versus their wild-type controls. The number and total time spent in seizure was reduced by $\sim 70\%$ ($p < 0.01$) in caspase-1 KO mice versus wild-type mice, resulting in decreased incidence of seizures during the recording period, but not in their average duration (see EEG trace in A). Spiking activity was not affected in caspase-1 KO versus wild-type mice.

Seizure onset in C57BL/6/SV129 mice (678 ± 71 s) was significantly delayed (threefold) by pralnacasan (25 μ g, icv, 45 min and 10 min before kainic acid), which also reduced the duration of seizures (97 ± 25 s) in mice by $\sim 70\%$ ($p < 0.01$). Pralnacasan did not affect any seizure

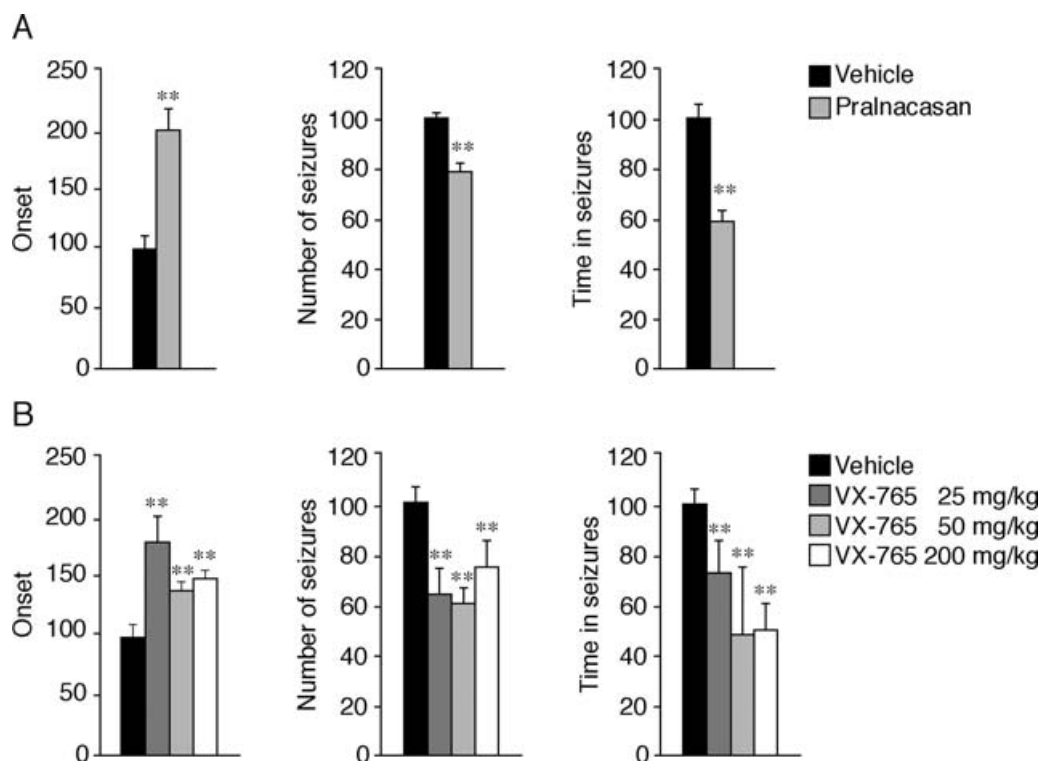


FIG. 2. Effects of caspase-1 inhibition on kainic acid-induced seizures in rats **A:** Pralnacasan ($2 \times 25 \mu\text{g}$ in $4 \mu\text{l}$ 20% cremophor in PBS, $n = 8$ rats) or its vehicle ($n = 7$) was injected intracerebroventricularly, 45 min and 10 min before the intrahippocampal injection of 40 ng kainic acid in $0.5 \mu\text{l}$ PBS. **B:** VX-765 (25, 50, and 200 mg/kg dissolved in 20% cremophor in PBS, $n = 8$ rats in each group) or its vehicle ($n = 15$) was injected intraperitoneally for 3 consecutive days. On day 4, rats received VX-765 or vehicle, 45 min and 10 min before the intrahippocampal injection of 40 ng kainic acid in $0.5 \mu\text{l}$ PBS. EEG activity was recorded continuously in freely moving rats for 30 min before (baseline) and for 180 min after the injection of the convulsant. Seizure parameters are defined in the Methods section. Data represent the mean \pm SEM of the various seizure parameters expressed as percentage of control values measured in vehicle-treated rats. Vehicles in pralnacasan- or VX-765-treated rats were pooled in a unique control group because they did not differ significantly [Onset (min): 7.9 ± 0.7 ; Number of seizures: 32 ± 1 ; Time in seizures (min): 44.0 ± 2.0 , $n = 22$]. ** $p < 0.01$ versus vehicle by one-way ANOVA followed by Tukey's test; statistical analysis was done on absolute values.

parameter in caspase-1 KO mice, supporting that its anti-convulsant activity was specifically mediated by caspase-1 inhibition (not shown).

DISCUSSION

The main finding of this work is that inhibition of caspase-1 in the brain provides significant protection from acutely induced seizures in rodents. This anticonvulsive effect was achieved either after intracerebral or after systemic administration of selective caspase-1 blockers and was associated with the ability of these drugs to prevent seizure-induced increase of the biologically active form of IL-1 β . The significant involvement of caspase-1 in seizures is strongly supported by the drastic reduction in seizure susceptibility observed in mice with deletion of the caspase-1 gene. Although wild-type and KO mice were bred separately, differences in their seizure susceptibility are unlikely because of the existence of different genetic backgrounds. Both the wild-type and KO mice originate from breeding couples of the same genetic background and derive from a similar number of genera-

tions. Moreover, reported differences in seizure susceptibility to kainic acid among mouse strains (22,23) concern specifically the mortality rate, behavioral seizures severity score, and neurodegeneration, and not the duration of seizures, which is the parameter significantly reduced in caspase-1 KO versus wild-type mice. Similarly, the onset to kainate-induced hippocampal EEG seizures is independent of the mouse strain as assessed in C57BL6, 129SV, and C57BL6x129SV mice (unpublished observation).

Caspase-1 inhibition or gene deletion significantly reduced ictal activity without changing interictal spiking; this effect was observed also after intrahippocampal injection of IL-1Ra or in mice overexpressing IL-1Ra in astrocytes (6,9). It is possible that a reduction in IL-1 β actions impairs the transition between interictal and ictal events, although some level of hippocampal hyperexcitability still persists. Similarly, classic AEDs such as phenytoin and carbamazepine provide seizure control without modifying interictal epileptiform activity in limbic structures (24).

The caspase-1 gene codes for a 45-kDa inactive precursor protein that is constitutively expressed in various cell types, including glia and neurons (25–27). This precursor

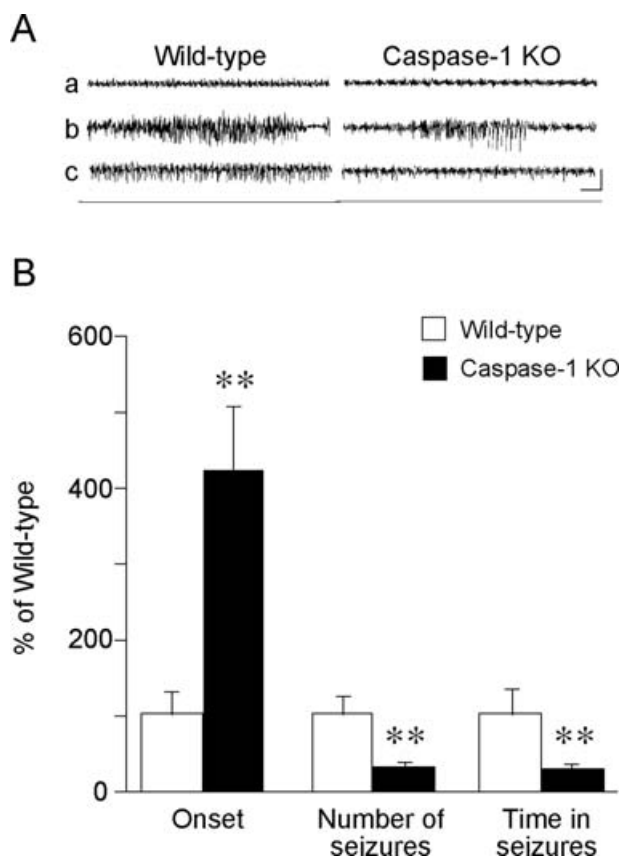


FIG. 3. Seizure susceptibility in caspase-1 knockout (KO) mice. Traces in **A** represent baseline recording (**a**, before kainic acid injection), typical ictal episode (**b**) and spiking activity (**c**), in wild-type and caspase-1 KO mice. EEG recordings were run continuously for 180 min after convulsant injection. Horizontal bar: 5 s. Bargrams in **B** show quantification of the EEG recordings. Data represent the mean \pm SEM ($n = 8-11$ mice in each experimental group) of the various seizure parameters expressed as percentage of the respective control values. Caspase-1 KO mice and their respective controls were injected in the hippocampus unilaterally with kainic acid (14 ng). * $p < 0.01$ versus wild-type mice by Student's *t* test. Statistical analysis of data was done on absolute values.

requires two internal cleavages before becoming the enzymatically active mature enzyme. Mature caspase-1 contributes to autoprocessing of its precursor protein by undergoing oligomerization with itself or other members of the caspase family, such as caspase-3 (28). Unlike other caspases, caspase-1 is specifically required for processing the inactive pro-IL-1 β and pro-IL-18 to active IL-1 β and IL-18, respectively (29). This process is followed by secretion of the mature cytokines from the cell through an as-yet-unknown mechanism.

In this study, pharmacologic inhibition of caspase-1 was achieved in rodent brain by using pralnacasan or VX-765, representing a new class of protease inhibitors that specifically inhibit caspase-1. Pralnacasan is a prodrug of the potent, selective, nonpeptide competitive inhibitor RU36384, and was the first caspase-1 inhibitor to enter

clinical development. VX-765 is a prodrug with improved oral bioavailability that is currently under clinical development for the treatment of inflammatory and autoimmune conditions without any ensuing signs of toxicity, as reported to date (12,14,30). The powerful anticonvulsant effect achieved in rats after peripheral administration of VX-765 opens the perspective of a clinical use of selective caspase-1 inhibitors for controlling seizures.

Our biochemical evidence indicates that both pralnacasan and VX-765 blocked the release of IL-1 β induced by activation of caspase-1 in organotypic hippocampal slice cultures, thus establishing a proof-of-concept that these drugs should block also the production and subsequent release of mature IL-1 β in vivo. In our experimental model of seizures, we indeed found that an anticonvulsant dose of pralnacasan led to a 50% reduction in the hippocampal levels of the 17-kDa mature form of IL-1 β induced by seizures (1,3,4,7,25). However, residual IL-1 β still remained in the hippocampus in the absence of caspase-1 activity, suggesting the existence of a pool with a slow turnover rate. Moreover, other enzymes such as matrix metalloproteinases, elastase, cathepsins, trypsin, or chymotrypsin, are able to process to some extent pro-IL-1 β , and this might also be the reason for the residual IL-1 β levels in pralnacasan-treated rats (31-33). In this respect, we found that caspase-1 inhibition does not affect CA3 pyramidal cell loss in kainate-injected rat hippocampi. It might be that the residual pool of mature IL-1 β is sufficient to sustain kainate toxicity in spite of significant seizure reduction. Indeed, it has been reported that kainate-induced damage in CA3 depends on a direct neurotoxic action of kainate rather than on seizures per se (34,35). Alternatively, kainate-induced cell loss may be insensitive to the proneurotoxic actions of IL-1 β which have been demonstrated so far on NMDA and AMPA-mediated toxicity only (36-38).

Our findings strongly suggest that the reduction of IL-1 β levels induced by pralnacasan or VX-765 is responsible for the anticonvulsive effects of these caspase-1 inhibitors in rodents. We cannot exclude that these drugs also reduced the production of IL-18 in the brain. However, we could not detect measurable levels of this cytokine in hippocampal homogenates either in control conditions or during the acute phases of seizures.

Some antiinflammatory treatments have been shown to reduce seizures in experimental models (39-41) and in some instances in humans (42), supporting the concept that inflammation in the brain is significantly implicated in the mechanisms of hyperexcitability. Of particular interest for seizure phenomena are the functional interactions between IL-1 β and glutamate-mediated neurotransmission. Thus IL-1 β increases glutamate release (43), inhibits its reuptake by glia (44), and enhances NMDA-mediated calcium influx into pyramidal neurons by inducing tyrosine phosphorylation of NR2B subunit (36).

These actions anticipate a proexcitatory effect of this cytokine and support its proconvulsant actions as described in vivo (3,4,6,10).

Clinical evidence linking IL-1 β to epilepsy also exists: caspase-1 and IL-1 are elevated in temporal lobe tissue of patients with intractable epilepsy (45–47) and a polymorphism in the promoter region of the IL-1 β gene is associated with therapy-resistant epilepsy (48,49). This polymorphism, when present in homozygotes, appears to predispose to an enhanced production of IL-1 β . Moreover, prominent inflammation in brain has been demonstrated in disease states associated with the subsequent development of intractable seizures (50,51).

Because IL-1 β exacerbates seizures and neuronal loss after various injuries, inhibits neurogenesis, and increases the blood–brain barrier permeability (38,52–54), pharmacologic approaches specifically targeted to block the overproduction of IL-1 β or its function(s) in diseased conditions may represent new, nonconventional strategies for the treatment of seizure disorders.

Acknowledgment: This work was supported by Fondazione Mariani Onlus, Milano, and Vertex Pharmaceuticals, Inc, Cambridge, MA, U.S.A. (to A.V.) and by Medical Research Council (to S.A.). Caspase-1 knockout mice were kindly supplied by Dr. W. Wong (BASF Bioresearch Corporation, Worcester, MA, U.S.A.).

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